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Age-related changes in collagen synthesis and degradation in rat tissues

Importance of degradation of newly synthesized collagen in regulating collagen production

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During developmental growth, collagens are believed to be continuously deposited into an extracellular matrix which is increasingly stabilized by the formation of covalent cross-links throughout life. However, the age-related changes in rates of synthetic and degradative processes are less well understood. In the present study we measured rates of collagen synthesis *in vivo* using a flooding dose of unlabelled proline given with [^{14}C]proline and determining production of hydroxy[^{14}C]proline. Degradation of newly synthesized collagen was estimated from the amount of free hydroxy [^{14}C]proline in tissues 30 min after injection. Collagen fractional synthesis rates ranged from about 5%/day in skeletal muscle to 20%/day in hearts of rats aged 1 month. At 15 months of age, collagen fractional synthesis rates had decreased markedly in lung and skin, but in skeletal muscle and heart, rates were unchanged. At 24 months of age, synthesis rates had decreased by at least 10-fold in all tissues, compared with rates at 1 month. The proportion of newly synthesized collagen degraded ranged from 6.4 ± 0.4% in skin to 61.6 ± 5.0% in heart at 1 month of age. During aging the proportion degraded increased in all tissues to maximal values at 15 months, ranging from 56 ± 7% in skin to 96 ± 1% in heart. These data suggest that there are marked age-related changes in rates of collagen metabolism. They also indicate that synthesis is active even in old animals, where the bulk of collagens produced are destined to be degraded.

INTRODUCTION

Collagens of various types are the most abundant proteins in vertebrates, representing approximately one-third of body protein in man (Harkness *et al.*, 1958; Picou *et al.*, 1966). They are found in the extracellular matrix and basement membranes of nearly all tissues, where their primary role is to provide a supportive extracellular framework for cells (Eyre, 1980). Other functions include platelet adhesion and aggregation [for a review, see Barnes (1985)], cell attachment (Kleinmann *et al.*, 1981), chemotaxis (Postlethwaite *et al.*, 1978), filtration in basement membranes [for a review, see Minor (1980)] and a role in morphogenesis and development [for a review, see Reddi (1984)]. The principal steps in collagen biosynthesis are well characterized [for reviews, see Prockop *et al.* (1979*a*,*b*) and Kivirikko & Myllyla (1987)] and alterations in these pathways have been implicated in a number of disease states, such as atherosclerosis, liver cirrhosis, pulmonary fibrosis, systemic sclerosis, rheumatoid arthritis, oesteogenesis imperfecta and Ehlers–Danlos Syndrome [for reviews, see Nimni (1983), Prockop & Kivirikko (1984) and Laurent *et al.* (1988)]. Although in some inherited disorders specific molecular lesions have been identified in collagen genes, in fibrotic and degenerative disorders altered regulation of collagen synthesis and/or degradation is likely to be of importance. For this reason an appreciation of the normal rates of collagen biosynthesis in tissues may lead to a greater understanding of the metabolic imbalance which may underlie or contribute to these conditions.

Studies of collagen metabolism in whole animals have been restricted because of the lack of suitable methods to assess rates of synthesis and degradation [for a review, see Laurent (1987)]. Early investigators used pulse-labelling methods (Neuberger *et al.*, 1951; Robertson, 1952; Neuberger & Slack, 1953; Thompson & Ballou, 1956) that did not take account of re-utilization of the labelling amino acid, giving apparently lower synthesis rates. Methods which reduced reutilization, namely by pulse–chasing (Nissen *et al.*, 1978), correcting for reutilization (Sodek & Ferrier, 1988) or the use of non-reutilizable labels (Jackson & Heininger, 1974, 1975; Molnar *et al.*, 1986), have suggested higher turnover rates than have the earlier methods. Application of 'continuous-infusion' methods, which aimed to minimize re-utilization and measured rates over comparatively short times, also indicated more rapid rates which were similar to values obtained with non-re-utilizable labels (Laurent *et al.*, 1978; Robins, 1979; Palmer *et al.*, 1980). The development of 'flooding-dose' methods for measuring protein-synthesis rates (McNurlan *et al.*, 1979; Garlick *et al.*, 1980) and its application to collagen (Laurent, 1982) confirmed rapid turnover rates for collagen in a variety of species and tissues (Schneir *et al.*, 1986; McAnulty & Laurent, 1987; Murray & Parkins, 1987, 1988; Opsahl *et al.*, 1987; Pell & Bates, 1987).

Collagen levels are determined by the balance between synthesis and degradation. Several studies of collagen biosynthesis suggested a decreased rate with aging (Neuberger *et al.*, 1951; Pierce *et al.*, 1967; Uitto, 1970; Bradley *et al.*, 1974), but there have been very few studies of age-related changes in collagen degradation, a process believed to occur both intracellularly (Bienkowski *et al.*, 1978; Imberman *et al.*, 1982; McAnulty & Laurent, 1987) and extracellularly (Lapière, 1965; Stricklin & Hibbs, 1988). Intracellular degradation has been reported to account for the fate of approximately one-third of newly synthesized collagen in the lung (Bienkowski *et al.*, 1978; Laurent & McAnulty, 1983), and the extent of the process varies from tissue to tissue (McAnulty & Laurent, 1987). There has been one

Abbreviation used: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

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report of the age-related changes in this process, which indicated that its magnitude remained unaltered in rabbit lungs between 1 week and 6 months of age (Bienkowski *et al.*, 1978).

In the present study we investigated collagen metabolism *in vivo* in rats aged between 1 and 24 months, using flooding-dose methods that we validated for use in older animals. The results suggest that changes in synthesis and degradation follow different patterns in various tissues and that degradation plays an important role in determining collagen production.

EXPERIMENTAL

Materials

L-[U-^{14}C]Proline (Amersham International, Amersham, Bucks., U.K., and New England Nuclear, du Pont, Stevenage, Herts., U.K.) was purified immediately before use by cation-exchange chromatography (Dowex 50W-X8 resin; 200–400 mesh; column dimensions 10 mm × 500 mm). The column was eluted with HCl (1.5 M) to separate proline from any radiolabelled contaminants (McAnulty & Laurent, 1987).

Animals

Experiments were undertaken on male Lewis rats (original source Bantin and Kingman, Aldbrough, Hull, Humberside, U.K.), obtained from our own breeding colony. Animals were housed in cages containing similar numbers of rats and fed *ad libitum* (the feedstuff used was Rat and Mouse Number 1, Modified Maintenance Diet from Scientific Diet Services, Witham, Essex, U.K.). They were regularly inspected, and only those showing no signs of disease were used in experiments.

Assessment of time course of a flooding dose of [^{14}C]proline

The time course of the flooding dose was assessed in young (1 month) and old (15 months) animals. Each animal was injected with L-[U-^{14}C]proline (5 μCi/100 g body wt.) with a flooding dose of L-proline (1.4 mmol/100 g) in phosphate-buffered saline (0.02 M-sodium phosphate/1.7 M-NaCl, pH 7.2; 0.5–1.5 ml), either intraperitoneally (15 months) or intravenously (1 month) in the lateral tail vein (McAnulty & Laurent, 1987). Animals were killed at one of three times after injection (5, 30 or 60 min) by ethoxyethane inhalation. All experiments were undertaken at the same time of day (11.00–14.00 h). The abdomen and chest were opened by midline incision, and blood was collected by cardiac puncture into heparinized tubes. The aorta was cut in the abdomen, and the heart and lungs perfused with ice-cold phosphate-buffered saline (10 ml) via the right ventricle. The heart and lungs were excised, then trimmed to remove blood vessels and major airways. A sample of abdominal skin was removed and skeletal muscle (gastrocnemius) dissected from the hind legs. Immediately after removal, all tissues were weighed and frozen in liquid N_2. Blood was centrifuged (2000 *g*, 15 min), plasma retained and all samples stored at −40 °C. Before further analyses the lungs and skeletal muscle were powdered at solid-CO_2 temperatures.

Age-related changes in collagen metabolism

Five groups of animals were used, aged 1 month (35 days), 2 months (66 days), 6 months (182 days), 15 months (468 days) and 24 months (742 days). Animals were injected with L-[U-^{14}C]proline (dose between 45 μCi and 170 μCi/100 g body wt.) with a flooding dose of unlabelled L-proline (1.4 mmol/100 g body wt.) in phosphate-buffered saline (0.5–1.5 ml), either intraperitoneally or intravenously in the lateral tail vein (McAnulty & Laurent, 1987). Animals were killed 30–40 min later, and the tissues were dissected as described above.

Analyses of tissues

The whole tissue or a powdered sample (0.8 g) was homogenized in ice-cold aq. 67% (v/v) ethanol using an Ultra-Turrax homogenizer (Marathan Laboratory Supplies, Kensal Green, London N.W.10, U.K.). Proteinaceous material was precipitated overnight at 4 °C (McAnulty & Laurent, 1987). This material was centrifuged (15000 *g*, 30 min, 4 °C), and the pellet was resuspended in aq. 67% (v/v) ethanol (3 ml) and re-centrifuged. This procedure was repeated once more. Supernatants were retained each time, pooled, filtered [0.65 μm filter; Millipore (U.K.) Ltd., Harrow, Middx., U.K.] and evaporated to dryness by using either a Speedvac concentrator (Savant, Hicksville, NY, U.S.A.) for lung or a Buchler Evapomix (Arnold R. Horwell, London N.W.6, U.K.) for other tissues. The residue was redissolved in water (1.5 ml).

Portions of the concentrated supernatant (tissue-free pool) were hydrolysed in 2 ml of 6 M-HCl at 110 °C for 16 h. The hydrolysates were decolorized with activated charcoal (0.08 g; Hopkin and Williams, Chadwell Heath, Essex, U.K.), filtered [0.65 μm-pore-size filter; Millipore (U.K.) Ltd.], and hydroxyproline was isolated from the hydrolysed supernatants by cation-exchange chromatography (Dowex 50W-X8 resin; 200–400 mesh; column dimensions 10 mm × 500 mm). The column was eluted with 1 M-HCl (Laurent *et al.*, 1985). The proteinaceous pellets were dried over anhydrous phosphorus pentoxide *in vacuo*, hydrolysed overnight in HCl and decolorized with charcoal.

The proline and hydroxyproline contents, total radioactivity and specific radioactivities were measured in the total proteinaceous hydrolysate and in both the hydrolysed and unhydrolysed supernatants (Laurent, 1982; Laurent *et al.*, 1982). This was achieved by oxidizing samples with chloramine-T and extracting the products into methylbenzene. Portions of these extracts were used to determine the radioactivity associated with proline and hydroxyproline; molar amounts of substance were determined from the absorbance of Δ_1-pyrroline and pyrrole, oxidation products of proline and hydroxyproline respectively.

Collagen fractional synthesis rates

Collagen fractional synthesis rates (k_s; %/day) were calculated as described previously (Laurent, 1982) using the equation:

$$k_s = \frac{S_B}{S_A t} \times 100$$

where S_B is the specific radioactivity of total hydroxyproline, both in the proteinaceous material and in the tissue-free pool, S_A is the specific radioactivity of proline in the tissue-free pool (precursor pool for protein synthesis) and t is time between injection and death in days. Thus, because of the interval between the time of death (when the animal was first opened by midline incision) and freezing, some further metabolism may have taken place which would result in a slight overestimate in the fractional synthesis rate. However, the dissection process was consistent, and rates in tissues at different ages will be comparable.

Determination of degradation of newly synthesized collagen

Degradation of newly synthesized collagen (%) was calculated from the relative proportions of hydroxy[^{14}C]proline in the tissue-free pool and proteinaceous fractions, as previously described (Bienkowski & Engels, 1981).

In several experiments, levels of free hydroxy[^{14}C]proline were analysed by h.p.l.c. as well as by chloramine-T oxidation. Hydrolysed supernatants, from both unlabelled lung tissue 'spiked' with hydroxy[2-^{14}C]proline (Amersham International)

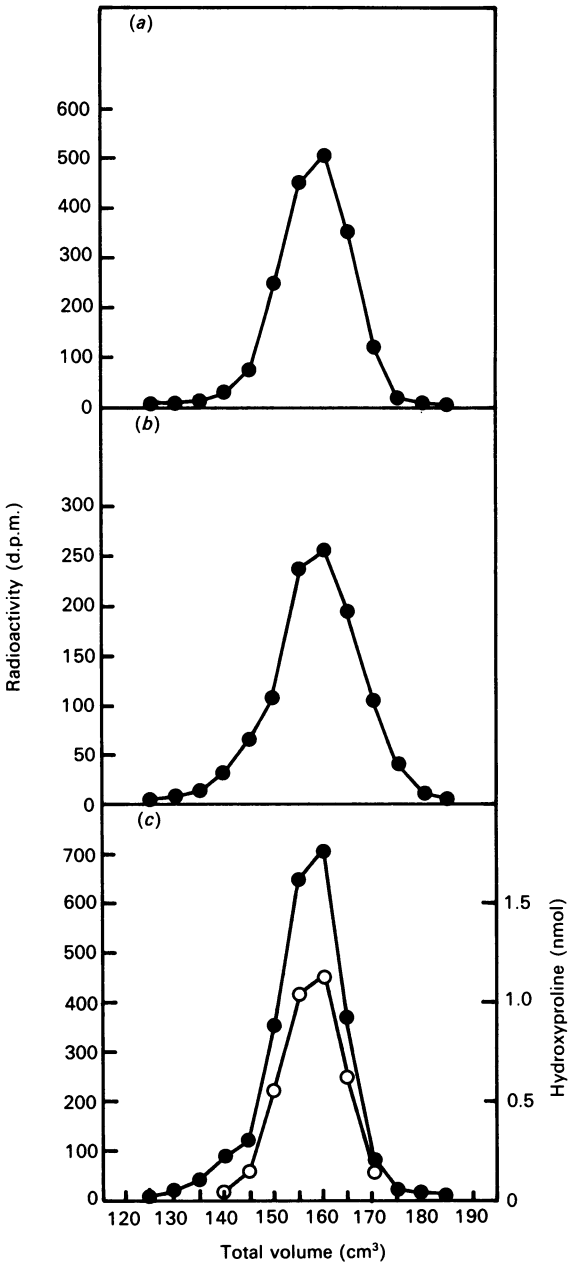

Fig. 1. Evaluation of degradation of newly synthesized collagen by an independent chromatographic system

Lung samples from 15-month-old rats were prepared for analysis as described in the Experimental section. Hydrolysed supernatants were subjected to cation-exchange chromatography, and fractions around the hydroxyproline peak were analysed by either chloramine-T oxidation or pre-column derivatization with NBD-Cl and separation by h.p.l.c. (*a*) A 'blank' lung sample 'spiked' with hydroxy-L-[2-^{14}C]proline. Radioactivity was extracted for each fraction after oxidation with chloramine-T. (*b*) Lung sample from animals injected with [^{14}C]proline. Radioactivity extracted per fraction, after oxidation with chloramine-T. (*c*) Identical lung sample to that in (*b*). Radioactivity (●) and the amount of hydroxyproline associated with each fraction (○), after pre-column derivatization with NBD-Cl and separation by reverse-phase h.p.l.c.

and labelled with [^{14}C]proline *in vivo*, were subjected to cation-exchange chromatography to isolate hydroxyproline (McAnulty & Laurent, 1987). Fractions in the region where hydroxyproline is eluted were retained, and each fraction was assayed by using either chloramine-T oxidation or h.p.l.c. The methods for chloramine-T oxidation are described elsewhere (Laurent *et al.*, 1982). Before separation by h.p.l.c., fractions were dried, reconstituted in water (50 μl) and potassium tetraborate buffer (50 μl) and allowed to react in the dark with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl; 12 mM; 50 μl) for 20 min at 37 °C. The reaction was quenched with 1.5 M-HCl (25 μl), and prepared for injection on to a reverse-phase C_{18} h.p.l.c. column by the addition of sodium acetate (50 mM, pH 6.4) in aq. 27% (v/v) acetonitrile (75 μl). This solution was filtered [0.45 μm-pore-size filter; Millipore (U.K.) Ltd.], and a portion (100 μl) was injected on to the h.p.l.c. column. The column was eluted with a gradient consisting of sodium acetate (50 mM, pH 6.4) in aq. 8% (v/v) acetonitrile and aq. 75% (v/v) acetonitrile, as described elsewhere (Campa *et al.*, 1990). The chromophore of hydroxyproline was detected at 495 nm, the eluate mixed with Scintillator 199 (Canberra-Packard, Hungerford, Berks., U.K.) and the radioactivity associated with hydroxyproline was determined. The corresponding profiles of radioactivity and amount of substance are shown in Fig. 1. It was apparent that hydroxy[^{14}C]proline determined by chloramine-T oxidation followed a pattern identical with that determined by h.p.l.c. and that both techniques detected radiolabelled hydroxyproline alone.

Statistical methods

All data are presented as means ± S.E.M. unless stated otherwise. The time-course experiments for the incorporation of [^{14}C]proline into proteinaceous material were analysed by multiple-linear regression (Swinscow, 1980). Age-related changes in collagen metabolism were analysed by ranking the data and applying the Wilcoxon–Mann–Whitney U-Test (Kraft & von Eeden, 1968), comparing with the previous age group only. Differences were considered to be statistically significant if the probability (P) value was less than 5% ($P < 0.05$).

RESULTS

The work described here represents part of a larger study of aging in male Lewis rats *in vivo*. Information relating to animal and organ weights, survival rates and collagen concentrations has been given in previous publications (Mays *et al.*, 1988*a*,*b*; Mays *et al.*, 1989).

Table 1 shows the time course for changes in tissue-free pool proline specific radioactivity after a flooding dose of proline, which was investigated in tissues from rats aged 1 and 15 months. The tissue-free-pool proline specific radioactivities were similar between 5 and 60 min after a flooding dose of [^{14}C]proline. Table 2 shows that the incorporation of [^{14}C]proline into proteinaceous material was essentially linear in all tissues over 60 min after a flooding-dose injection.

Table 3 shows the ratios of the tissue-free pool proline specific radioactivities compared with the specific radioactivity in the injection fluid from the tissues in the experiment examining age-related changes in collagen metabolism. Although the specific radioactivity of the injection fluid varied, depending on the absolute amount of radioactivity given to each group, the specific radioactivities of the free pool were at least 70% of that of the injected solution and in many instances greater than 90%. The largest discrepancy between the injection fluid and the tissue-free pool was in 24-month-old muscle.

Table 4 shows collagen fractional synthesis rates, representing both proteinaceous and rapidly degraded collagen (i.e. total collagen), based on free and proteinaceous hydroxy[^{14}C]proline. Collagen synthesis rates decreased over the period studied. Between 1 and 24 months of age, synthesis rates decreased at least 10-fold in heart, lung and skeletal muscle, whereas for skin

Table 1. Time-related changes in the specific radioactivity of [^{14}C]proline in tissue-free pool of plasma, heart, lung, skeletal muscle and skin of young and old rats

Rats, 1 month old rats (body wt. 119±7 g; $n = 9$) and 15 months old (568±20 g; $n = 9$) were killed 5, 30 or 60 min after receiving either an intravenous (1 month old) or intraperitoneal (15 months old) injection of [^{14}C]proline (5 μCi/100 g body wt.) with a flooding dose of proline (1.4 mmol/100 g) in phosphate-buffered saline (0.5–1.5 ml) as described in the Experimental section. The proline specific radioactivities in tissue-free pool were measured as described in the Experimental section. All values are means±S.E.M. for three animals at each time point.

		10^{-4} × Proline tissue-free-pool specific radioactivity (d.p.m./μmol)					
	Age of rats (months)...	1			15		
Tissue	Time after injection (min)...	5	30	60	5	30	60
Plasma		1.62±0.04	1.75±0.06	1.81±0.28	1.41±0.03	1.61±0.27	1.55±0.09
Heart		1.40±0.31	1.82±0.18	1.53±0.27	1.21±0.02	1.28±0.19	1.17±0.08
Lung		1.93±0.15	1.68±0.09	1.99±0.07	1.55±0.22	1.29±0.07	1.77±0.02
Muscle		1.15±0.02	1.31±0.16	1.33±0.06	1.04±0.02	1.00±0.05	1.16±0.02
Skin		1.18±0.08	1.54±0.08	1.17±0.06	1.11±0.13	0.63±0.16	0.97±0.13

Table 2. Time-related changes in the specific radioactivity of proteinaceous [^{14}C]proline in heart, lung, skeletal muscle and skin

In the same animals as in Table 1, the incorporation of [^{14}C]proline into proteins was assessed as described in the Experimental section. All values are means±S.E.M. for three animals at each time point; r is the correlation coefficient.

		Specific radioactivity of proteinaceous proline (d.p.m./μmol)							
	Age of rats (months)...	1				15			
Tissue	Time after injection (min)...	5	30	60	r	5	30	60	r
Heart		11±1	76±5	159±9	0.99	7±1	30±1	55±7	0.96
Lung		9±1	82±4	186±2	0.99	7±1	32±4	55±4	0.97
Muscle		12±2	60±8	119±12	0.97	12±1	21±4	60±18	0.78
Skin		6±2	60±10	114±18	0.91	2±1	6±1	12±1	0.96

Table 3. Specific radioactivity of [^{14}C]proline in injection fluid and ratio of tissue-free pool proline specific radioactivities compared with injection fluid specific radioactivities

Rats were killed and tissues processed as described in the Experimental section. Specific radioactivities of the injection fluids are the means of three measurements for each age group, n is the number of animals in each age group. Results are means±S.E.M.

			Tissue-free-pool proline specific radioactivity / Injection fluid proline specific radioactivity			
Age (months)	n	10^{-3} × Proline specific radioactivity in injection fluid (d.p.m./μmol)	Heart	Lung	Muscle	Skin
1	8	193.0	1.00±0.05	0.96±0.05	0.94±0.06	0.74±0.06
2	4	83.0	0.70±0.06	1.00±0.06	0.85±0.04	0.84±0.10
6	6	146.6	0.95±0.05	0.88±0.04	0.96±0.06	0.87±0.03
15	6	277.4	0.97±0.01	0.94±0.01	0.79±0.03	0.84±0.05
24	6	215.4	0.99±0.04	1.04±0.02	0.69±0.07	0.91±0.03

they decreased 25-fold. In both cardiac and skeletal muscle there were increases in synthesis rates between 6 and 15 months of age.

The extent of degradation of newly synthesized collagen, based upon the appearance of free hydroxy[^{14}C]proline, is shown in Table 5. This proportion increased in all tissues between 1 month and 15 months of age. The proportion degraded fell between 15 and 24 months of age in heart, lung and skin.

DISCUSSION

General methodological considerations

This study applied 'flooding-dose' methods developed previously (Laurent, 1982; McAnulty & Laurent, 1987) to examine age-related changes in collagen metabolism. Here we have shown that these methods were effective in young and old animals, since

Table 4. Age-related changes in collagen fractional synthesis rates

Collagen fractional synthesis rates were measured as described in the Experimental section. Each value is expressed as the mean ± S.E.M. Statistical significance compared with previous age group only: $*P < 0.05$. n is the number of animals in each group.

Age (months)	n	Tissue...	Gross fractional synthesis rate (%/day)			
			Heart	Lung	Muscle	Skin
1	8		19.21 ± 2.50	13.51 ± 0.54	4.93 ± 0.43	12.05 ± 1.64
2	4		6.16 ± 2.42*	8.96 ± 0.67*	1.33 ± 0.16*	2.37 ± 0.22*
6	6		8.94 ± 1.58	4.21 ± 0.51*	1.62 ± 0.33	0.86 ± 0.11*
15	6		15.03 ± 4.71	4.69 ± 0.36	3.75 ± 0.37*	0.96 ± 0.11
24	6		2.08 ± 0.63*	0.97 ± 0.14*	0.52 ± 0.14*	0.44 ± 0.03*

Table 5. Age-related changes in the degradation of newly synthesized collagen

The proportion of newly synthesized collagen degraded was calculated as described in the Experimental section. Results are means ± S.E.M. Statistical significance compared with previous age group only: $*P < 0.05$. n is the number of animals in each group.

Age (months)	n	Proportion of newly synthesized collagen degraded (%)			
		Heart	Lung	Muscle	Skin
1	8	61.5 ± 5.1	27.6 ± 3.2	26.2 ± 2.1	6.4 ± 0.4
2	4	56.7 ± 4.6	35.1 ± 2.5	44.8 ± 4.7*	8.4 ± 0.5*
6	6	93.4 ± 0.6*	69.1 ± 1.5*	78.0 ± 7.0*	14.3 ± 3.5
15	6	96.2 ± 0.9	82.3 ± 1.1*	95.3 ± 0.6	56.3 ± 7.0*
24	6	84.6 ± 3.8*	62.3 ± 5.7*	90.6 ± 2.7	29.3 ± 2.0*

the tissue-free pool specific radioactivity remained relatively constant over the labelling period, and incorporation of [^{14}C]proline into proteinaceous material was linear with respect to time (Tables 1 and 2). One criterion for flooding is that the specific radioactivity of the precursor amino acid in the tissue-free pool is close to that in nascent proteins. In general this has not been demonstrated, but in one previous study we have shown that the proline specific radioactivity in rabbit skin procollagen type I was equivalent to the specific radioactivity in the free pool for this tissue, suggesting that the tissue-free pool value represented the precursor pool for collagen synthesis (Laurent, 1982).

The use of tissue-free pool specific radioactivity as a measure of the precursor pool has some limitations, most notably owing to compartmentation of precursor amino acid pools both *in vivo* when using continuous infusion (Ilan & Singer, 1975; Fern & Garlick, 1976; Robins, 1979) and *in vitro* (Hildebran *et al.*, 1981; Low *et al.*, 1986). Proline is known to be synthesized *de novo* from glutamate and ornithine, the latter being then preferentially incorporated into collagen from those sources (Bellon *et al.*, 1987; Opsahl & Ehrhart, 1987). Similarly, proline derived from protein degradation may be incorporated into proteins (Jackson & Heininger, 1974, 1975). Both *de novo* synthesis and re-utilization will dilute the specific radioactivity of the precursor pool for collagen synthesis. However, the flooding dose produced a concentration of free proline that has been shown *in vitro* to limit synthesis of proline *de novo* (Opsahl & Ehrhart, 1987) and it should also provide an overwhelming source of exogenous proline to reduce reutilization. Thus the flooding dose may overcome some of the apparent compartmentation of amino acid precursors. Even if the tissue-free-pool proline specific radioactivities were not equivalent to the true precursor pool specific radioactivity, since there were minimal variations in the kinetics of tissue-free proline at different ages (Table 1), then any errors in this estimate should be consistent and synthesis rates would be comparable between tissues at different ages.

A further limitation of the flooding-dose method is that the large dose of proline may exert an effect on collagen synthesis rates. However, this is unlikely, since the flooding dose of proline does not alter total protein synthesis rates (Mays, 1990), and the concentration of proline produced by the flooding dose is known not to affect collagen synthesis rates *in vitro* (Opsahl & Ehrhart, 1987). Moreover, there were no differences in muscle net collagen synthesis rates measured *in vivo* using either continuous infusion (i.e. low-dose proline; Laurent *et al.*, 1978) or flooding dose (i.e. high-dose proline; Laurent *et al.*, 1985).

A common assumption in studies of collagen turnover is that the bulk of measured hydroxyproline derives from collagen (Bradley *et al.*, 1974; Bienkowski *et al.*, 1978; Etherington & Bailey, 1982; Murray & Parkins, 1987, 1988; Opsahl *et al.*, 1987; Pell & Bates, 1987). It is known that other proteins contain hydroxyproline, including elastin (Rosenbloom, 1987), the C1q component of complement (Porter & Reid, 1978), acetylcholinesterase (Mays & Rosenberry, 1981), conglutinin (Strang *et al.*, 1986) and an apolipoprotein of surfactant (Patthy, 1987). However, these compounds probably contribute negligibly to the total hydroxyproline content of tissues, and, unless they turnover very rapidly, measurements based on the turnover of tissue hydroxyproline probably represent collagen metabolism. Currently there are sparse data concerning the turnover rates of these proteins, and none are available from contemporary '*in vivo*' methods. Their contributions can only adequately be assessed when such data are obtained.

The methods used here assume that proline hydroxylation only occurs post-translationally. In cell-free studies, Trelstad *et al.* (1981) proposed a process for free-radical hydroxylation of proline. Such a mechanism, if active in the present system, would affect determination of hydroxyproline. However, this process

has been suggested not to play a role for cells in culture (Bienkowski, 1984) and may be unlikely to do so *in vivo*. Variations in the extent of proline hydroxylation are also unlikely to affect our measurements, given the observation that the level of proline hydroxylation of intact collagens does not alter with age (Barnes *et al.*, 1974).

The methods for measuring degradation of newly synthesized collagen *in vivo* have been detailed elsewhere (Laurent & McAnulty, 1983; McAnulty & Laurent, 1987). However, because of the high proportions of degradation reported here and the extremely high radioactivity associated with the free hydroxyproline, we undertook an experiment to ensure that the radioactivity determined was indeed associated with hydroxyproline. This involved assaying individual fractions from cation-exchange chromatography, either using chloramine-T oxidation or h.p.l.c. with pre-column derivatization with NBD-Cl. This demonstrated that the profile of radioactivity associated with the hydroxy[^{14}C]proline peak from the initial cation-exchange chromatography determined by either chloramine-T oxidation or h.p.l.c. were similar. It is thus highly unlikely that the hydroxy[^{14}C]proline was contaminated with other radiolabelled compounds, since two independent methods, in which separation is based on different properties of the compounds, give such similar data.

The actual source of free hydroxyproline has not been demonstrated by either ourselves or others, although currently the bulk of the data suggests that it arises from the degradation of newly synthesized collagen (Bienkowski *et al.*, 1978). In the present study the time course of the appearance of free hydroxy[^{14}C]proline was not investigated; however, previously we have shown that, in rat tissues, free hydroxy[^{14}C]proline was not lost significantly into the circulation over 30 min (McAnulty & Laurent, 1987). Thus free hydroxy[^{14}C]proline measured in tissues was most likely to have arisen in that tissue from degradation of hydroxyproline-containing proteins, principally collagen (see above).

Age-related changes in collagen metabolism

Values reported here for collagen synthesis rates were much higher than in previous studies (Neuberger *et al.*, 1951; Robertson, 1952; Neuberger & Slack, 1953; Lindstedt & Prockop, 1961; Pierce *et al.*, 1967; Niedermuller *et al.*, 1977), but the general trend for an age-related decrease in collagen turnover (Neuberger *et al.*, 1951; Lindstedt & Prockop, 1961; Niedermuller *et al.*, 1977) was a consistent finding. This age-related decrease was observed in all tissues; however, a continual decline was not observed in all tissues. In heart and skeletal muscle, synthesis rates remained high, even in 15-month-old animals, although in these tissues degradation of newly synthesized collagen accounted for the bulk of all collagen synthesized and highlights the importance of accounting for this process in estimates of collagen synthesis.

The extent of degradation of newly synthesized collagen increased with age, so that, in cardiac and skeletal muscle, greater than 90% was rapidly degraded in older animals. However, the utilization of this pathway varied between tissues; for instance, only 5% was rapidly degraded in young skin, whereas more than 50% of all collagens synthesized in heart at any age were rapidly degraded.

Intracellular degradation has been proposed to play a role in regulating collagen production (Berg, 1986; Laurent, 1987). In addition, a role in eliminating structurally detective collagen has been suggested (Berg *et al.*, 1980), and a basal level has been ascribed to 'metabolic noise' (Bienkowski, 1985). It seems unlikely that degradation at the high levels seen here was due entirely to production of defective collagen, but this hypothesis still needs to be assessed. One possible determinant is the degree of post-translational hydroxylation. Under-hydroxylated collagens are known to be susceptible to degradation (Berg & Prockop, 1973; Berg *et al.*, 1983). Prolyl hydroxylase activity has been reported to decrease with age (Risteli & Kivirikko, 1976), although when examined in isolated collagens there were no changes in the extent of hydroxylation with age (Barnes *et al.*, 1974). Gene modifications should also be assessed. It is known that single base-pair deletions in genes can have dramatic effects on collagen stability (Baker *et al.*, 1989; Constantinou *et al.*, 1989). Clearly more information is required on age-related changes in transcriptional, translational and post-translational processes and their relationship to the magnitude of degradation of newly synthesized collagen.

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